Antenatal inflammatory insults and preterm brain injury

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Summary & general discussion

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Perinatal inflammatory stress and premature birth are strongly associated with neurological disabilities throughout life [1]. Over the last decades, extensive research has been performed to identify causes and unravel pathophysiological mechanisms, leading to injury of the developing brain in order to facilitate development of therapeutic strategies. Despite different approaches and numerous studies, many essential questions are still unanswered and the obtained knowledge resulted in only limited translation into clinical practice. As a result, still high rates of neurodevelopmental impairment are observed in survivors of prematurity. From the different essential questions that remain unanswered, I focused on the following items which formed the basis for the chapters in this thesis:

1. The peripheral and cerebral outcomes of the fetus following different intra amniotic microbial stimuli **(chapter 2-4).**
2. The time-dependent changes in the developing brain in the course of perinatal inflammatory stress **(chapter 2 & 3).**
3. The effect of repetitive inflammatory stimuli on preterm brain development **(chapter 2).**
4. Therapies for EoP with a focus on cell-based therapies and their region-dependent effects **(chapter 5 & 6).**
5. Defining the optimal window of opportunity for new neuroprotective interventions **(chapter 3 & 6).**

Perinatal brain injury (EoP) can result from different origins with different underlying mechanisms. I, therefore, studied the effects of chorioamnionitis **(chapter 2-4)** and global hypoxia-ischemia (HI) **(chapter 5-6)** separately as two important, independent, contributing factors to EoP. For these studies, we have primarily used two preclinical ovine models closely mimicking human brain development *in utero*. In **chapter 2-4**, ovine models of **intrauterine infection/inflammation** have been used to mimic acute or chronic chorioamnionitis with fetal involvement (FIRS) to study its effects

on fetal brain development. Considering the polymicrobial origin of chorioamnionitis different infectious/inflammatory triggers were used within these studies, including *Ureaplasma Parvum, C. albicans* and *E. coli*-derived Lipopolysaccharide (LPS) as representatives of most common bacterial and fungal isolates in chorioamnionitis [2].

In **chapter 2-4** we showed that intra amniotic exposure to LPS or *C. albicans* provoked an acute increase in fetal systemic IL-6 concentrations within the first days after *in utero* exposure. More recently, these findings were recapitulated by UP (unpublished data), indicating that regardless of the microbial trigger a systemic inflammatory response is induced within the same timeframe. Pro-inflammatory cytokines, and IL-6 in particular, contribute to the activation of cerebral endothelium and cells of the blood-brain barrier [3-5]. These cells in turn activate adjacent microglia and astrocytes as their primary targets and producers of inflammatory mediators, ultimately resulting in a neuroinflammatory response [6-10]. This is also supported by our data in which we showed that the induction of a systemic response was followed by the induction of a cerebral inflammatory response as shown by an increase in microglia density and subsequently cerebral injury **(chapter 2-4).** In addition, IL-6 inhibits the development of new neurons in experimental models which is part of the pathogenesis of hyper-excitable neurological conditions including epilepsy and autism spectrum disorders [3, 11].

The clinical importance of increased fetal circulatory IL-6 levels has been further underscored by studies which showed a strong association between IL-6 levels and neonatal morbidity and mortality [12]. Numerous clinical studies have shown that fetal involvement by the induction of FIRS (characterized by increased pro-inflammatory cytokines including IL-6) during chorioamnionitis is related to higher rates of neonatal sepsis and multi-organ adverse outcomes including perinatal brain injury [12, 13] and in particular CP [14]. A recent study including 2,390 extremely preterm infants (<27 weeks of gestation) demonstrated that the presence of clinical chorioamnionitis was associated with an increased risk of cognitive impairment at 18-22 months of corrected age [15]. In contrast, histological chorioamnionitis without fetal involvement has not been associated with adverse neurodevelopmental outcomes [16, 17] and shown to be only a weak risk factor for CP [16]. These combined data suggest that the release of inflammatory cytokines (FIRS) in the course of intrauterine infections

play a crucial role in the initiation and aggravation of brain injury [14]. Based on the items described above, it is appealing to consider systemic IL-6 levels as a prognostic marker to predict cerebral outcomes. However, caution is required for the following reasons: Increased plasma IL-6 levels are also found after non-infection-related insults including Rh-alloimmunization [18], trauma [19] and HI insults [20]. It still needs to be established whether the prognosis of FIRS differs if induced by infectious or non-infectious insults. Moreover, increased plasma levels of IL-6 in the acute phase (<24 h) following an ischemic insult or traumatic brain injury have been shown to be a reliable prognostic marker for adverse cerebral outcomes [21, 22]. We demonstrated that IL-6 concentrations were only acutely and transiently increased following intrauterine exposure to inflammation (UP, LPS) **(chapter 2 and 3)**. Therefore, the timing of IL-6 analysis appears to be essential as marker for cerebral prognosis and needs to be incorporated into the clinical decision model. Further studies should focus on the use of IL-6 in combination with other implemented systemic inflammatory markers (CRP, procalcitonin) or novel biomarkers that can determine the onset of intra amniotic infections [23] to better predict cerebral outcomes. Currently, promising biomarker studies are ongoing that meet this need in the future.

In women who deliver preterm, *Ureaplasma* species are the most frequently isolated microorganisms from the amniotic fluid and placentae [24]. Although *Ureaplasma parvum* (UP) is known as a pathogen of low virulence, it is associated with chorioamnionitis, spontaneous abortions/ miscarriages and neonatal respiratory diseases [25]. Despite the fact that these microorganisms have been routinely found within placentae of pregnancies with chorioamnionitis, the role of *Ureaplasma* species as a causative agent has not been irrefutably explained. The controversy surrounding the role of UP in disease is reinforced by the fact that not all women infected with *Ureaplasma* spp. develop chorioamnionitis [25, 26]. In **chapter 2** we however show that ***Ureaplasma parvum* (UP)** is not a harmless commensal colonizing the amniotic fluid. Chronic intra amniotic exposure induced changes in the fetal brain that might explain the observed altered neurological outcomes at 2 years of age in children born following chorioamnionitis by UP [27]. We found that chronic UP exposure in the third trimester decreased the number of astrocytes which was associated with increased number of oligodendrocytes (OLs) and epigenetic changes in the cerebral white matter and hippocampus. In line, changes in astrocyte function or density are associated with altered neurological

outcomes [28]. In particular, altered astrocyte protein expression (GFAP) and disrupted astrocyte maturation have been implicated in the pathogenesis of neurodevelopmental disorders such as autism and cerebral palsy. In addition, the increase of OL lineage cells, as seen following 42d of UP exposure, might indicate replenishment of OLs upon initial loss in the acute phase following UP exposure. This combined with the loss of MBP suggests a maturation arrest of OL progenitor cells which is a key feature of white matter injury in preterms [29, 30].

To gain more insight in the time dependent changes of the white matter in these fetal brains we studied the effects of intra amniotic LPS exposure over time. We found increased numbers of apoptotic cells in the white matter which was followed by loss of (pre-) OLs at 15 days post LPS exposure **(chapter 3)**. Such changes of the white matter predispose to the long-term adverse neurological outcomes in later life [30], which supports the developmental origin of health and disease (DOHaD) hypothesis. Considering the clinical importance, several potential mechanisms responsible for this altered white matter development, were studied and described in both **chapter 2 and 3**. First, aberrant or excessive microgliosis following LPS exposure was found **(chapter 3)** with subsequent cell death and/or maturation arrest of OLs with detrimental effects for the immature brain. Second, the observed alterations in astrocyte expression **(chapter 2)** is proposed to induce altered OL maturation since astrocytes are important for the metabolic support of OLs and key in regulating glutamate homeostasis [31]. At this stage of fetal brain development, pre-OLs are typically present in relative high numbers, and these cells are particularly vulnerable to glutamate receptor induced injury. When astrocyte function is altered, OLs lose metabolic support, glutamate homeostasis is disturbed and pre-OLs are prone to glutamate induced injury [32].

Alternatively, inflammation-induced epigenetic changes during early development can cause substantial lasting neurodevelopmental impairments later in life [33, 34]. The observed increase of the DNA methylation marker 5-mc **(chapter 2)**, an important repressor of gene transcription [35], is indicative for such epigenetic changes which are already induced during pregnancy complicated by chorioamnionitis. These findings highlight that essential alterations can occur in the antenatal period, which might be prerequisite for disturbed maturation and differentiation of immature OLs with potential detrimental consequences in later life. Based on

the data described in this thesis, longitudinal follow up studies in our pre-clinical models are ongoing in which the long term consequences of the pathophysiological processes imitated in the perinatal period will be studied causally. Such a longitudinal study enables us to study tertiary mechanisms of brain injury which include epigenetic changes and persistent inflammation [9]. These processes can persist for months or years after the initial insult in the perinatal period and are proposed to contribute to the prevention of endogenous repair and regeneration which contribute to the neuropathological substrate of EoP [9].

Chorioamnionitis is not limited to bacteria or bacterial products but also include viral and fungal species [2]. Although intra-amniotic fungal infections are rare, its consequences on fetal development are of enormous impact including a high mortality rate and severe neurological disabilities. We have chosen *C. albicans* infection since this is the most common fungal species found in women diagnosed with chorioamnionitis **(chapter 4)** [2]. As shown in this chapter, intra-amniotic exposure to *C. albicans* resulted in a systemic immune response (increased IL-6 levels) with concomitant microglial and astrocyte activation, focal white matter disturbances, increased cell death and fetal death within 5 days upon exposure without fungal invasion of the brain parenchyma. We have previously shown that *C. albicans* resulted in skin, lung [36] and gastro-intestinal tract inflammation [37]. Moreover, Nikiforou et al. [37] showed fungal translocation through the epithelial barrier within the blood stream from 3 days onwards. Since at this stage no invasive growth of *C. albicans* was present in other organs including the brain parenchyma, we consider that the systemic inflammatory response at this stage of the infection as most important trigger for cerebral inflammation and subsequent injury which corresponds with the postulated pathophysiological changes of the brain following intra-amniotic UP and LPS exposure.

Antenatal treatment for Candida chorioamnionitis is challenging and, until now, only resulted in half of cases in the delivery of living infants [38]. We showed that intra-amniotic Fluconazole treatment after intra-amniotic *C. albicans* infection successfully eradicated *C. albicans* from the CSF and temporarily inhibited systemic immune activation. However, modulation of the cerebral inflammatory response and prevention of the concomitant white matter injury was not observed. Nonetheless, a single dose of Fluconazole promoted fetal survival. In addition, Nikiforou et al.

showed that **Fluconazole** treatment was successful in decreasing colonization and epithelial injury of the fetal gut. Taken together, we consider that the decreased mortality is a result of the decreased colonization and epithelial injury of the fetal gut and potentially the lung resulting in a temporarily diminished systemic inflammatory response. Besides eradication of the microbe, future studies should aim at preventing the inflammation- and/or HI-induced adverse effects on the brain including immune-modulatory/regenerative therapies.

The etiology of EoP is complex and multifactorial. Over the last decade (pre)clinical evidence demonstrated that the brain experiences altered susceptibility when exposed to a second injurious hit following pre-exposure to inflammation [39, 40]. This concept of sensitization and preconditioning is supported by clinical data showing that the combination of antenatal infection and a hypoxic-ischemic (HI) insult around birth dramatically increases the risk of cerebral palsy (OR 78) when compared to either HI (OR 2.5) or infection (OR 7.2) alone [39]. Besides this specific combination of insults, multiple ante-, peri- or postnatal hits can contribute to the development of brain injury in the preterm infant including small for gestational age and impaired placental growth [41-43]. In addition, evidence is accumulating that postnatal ventilation-induced white matter injury, barotrauma around birth, (par)enteral feeding, necessary medication (glucocorticoids), surgeries, all could contribute to the development of EoP [44]. Clinically, exposure to multiple hits is associated with an enormous increase in the risk of and severity of white matter abnormalities [41, 45, 46]. Therefore, the impact of these factors on brain development also depends on interactions between different insults and treatments which need to be further explored.

In **chapter 2** we investigated the effect of multiple inflammatory hits, representing intrauterine inflammation and possible postnatal infections, and the effect on brain development. In this double-hit study, we found that chronic intra-amniotic UP exposure prevented microgliosis, epigenetic and lipid profile changes and myelin disturbances when animals were exposed to a second inflammatory hit with **lipopolysaccharide (LPS)** from E.coli. In line, this **'preconditioning'** phenotype was also found in ovine fetuses exposed to 70 days of UP and 7 days LPS [47]. Such preconditioning renders the brain less susceptible to a second insult, thereby resulting in less brain injury [48]. In contrast to these cerebral findings, UP infection for 24 days appeared to sensitize the fetal lungs [49] and gut (unpublished data) against a

second inflammatory hit with LPS indicating that target organ and duration of the insult determines susceptibility for a second hit. The underlying mechanisms by which inflammation establishes a favorable environment protecting the brain against a second event remain largely unknown. Gene ontology analysis reveals that the most over-represented genes belong to immune and inflammatory processes and cell death pathways in LPS-induced preconditioning in the immature brain [48]. Moreover, the time delay between insults appears to be critical in determining the pathological outcome as the same sublethal exposure can render the tissue either more (sensitization) or less (preconditioning) sensitive to the same subsequent, severe insult, depending on the interval between events. Mallard et al. showed that the vulnerability of the brain to an HI insult following *in utero* inflammation differs between neonatal and adult brains resulting in sensitization and preconditioning respectively [50]. Altogether, these data support the complexity of the underlying pathophysiological mechanisms of EoP. Considering the clinical heterogeneity between patients makes it almost impossible to develop tailor-made treatments and predict outcomes on individual levels. Recognition of individual causal factors leading to brain damage is the first step in a complex translational mission to tailor safe and effective therapies resulting in individualized/ personalized medicine.

Treatments

The introduction of therapeutic hypothermia as a treatment for moderate to severe neonatal encephalopathy follows two decades of pre-clinical studies in experimental HI models and clinical trials [51]. There is clear evidence that therapeutic hypothermia in this setting reduces adverse outcomes including decrease of mortality and neurodevelopmental disability at 18 months of age (RR 0.75% CI 0.68-0.83) which persists into childhood. However, around 40% of infants , have an adverse neurodevelopmental outcome, despite treatment [52]. Moreover, in preterm infants hypothermia is contra-indicated since it is associated with increased risk of side effects including poor neurodevelopmental outcome. Recently, the crucial role of the immune response has been recognized as having an important influence on outcome of hypothermia treatment [53-55]. It has been shown

that therapeutic hypothermia is ineffective and even harmful in the presence of infection/inflammation in adult clinical studies [55]. In a pre-clinical neonatal rodent study cooling was not neuroprotective in inflammation-sensitized HI [54]. In a small prospective study of placental histology being related to MRI of babies undergoing therapeutic hypothermia, therapeutic hypothermia was less protective in babies whose placentas showed chorioamnionitis [55]. Considering these data, the need for new therapeutic strategies to prevent EoP is emerging. As already elaborated in the introduction of this thesis, stem cell therapies are of increasing interest as a neurotherapeutic for neonatal brain injury and may show great potential in the prevention of encephalopathy of prematurity [56]. As shown in **chapter 2-4** the initiation of a cerebral inflammatory response is one of the essential steps leading to brain injury. Modulating this inflammatory response directly or indirectly is considered to be a key mechanism of action of a potential treatment. Second, regeneration of injured OLs and neurons and/or stimulate the maturation of these cells is a prerequisite of a new treatment to prevent EoP [9, 57]. Mesenchymal stromal cells possess both anti-inflammatory and regenerative properties. We have previously shown in an ovine model of HI-induced brain injury that intravenous administration of bone marrow-derived adherent stromal cells (MSCs, MAPCs) prevented cerebral inflammation, white matter injury and loss of function/decreased seizure activity following global hypoxia-ischemia [58, 59].

Besides the cerebrum, the cerebellum is increasingly appreciated as an important contributor in EoP [60]. Clinical data show that disturbed cerebellar development in prematurely born children can still be detected at school age/adolescence and is associated with adverse neurodevelopmental outcome [61]. More precisely, it has been shown that cerebellar injury plays an important role in the high prevalence of non-motor deficits like cognition, learning and behavior in survivors of prematurity. Therefore, protection of the cerebellum following preterm birth and/or HI is clinically highly relevant. In **chapter 5** we demonstrated that besides the cerebral white matter, MAPC therapy also protected the cerebellum against HI-induced injury. We found marked cortical injury, microgliosis and hypomyelination in the cerebellum following global HI, changes that are indicative as a pre-stage for cerebellar underdevelopment [60]. We showed that these cerebellar alterations were prevented by intravenous administration of MAPCs and potentially may prevent neurodevelopmental disorders in later life.

The different phases leading to brain injury following a HI insult have been well described and discussed in the introduction of this thesis [9, 62, 63]. Numerous studies have shown that microglia become excessively activated within 24 hours after an acute global HI insult and abundantly release pro-inflammatory cytokines, free oxygen species and excitatory amino acids [62-65]. Moreover, systemically, global HI also provokes a pro-inflammatory environment which triggers systemic release of danger signals reaching peak values around 24 hours after the insult [62-64, 66]. This systemic accumulation of DAMPs has been associated with massive activation of the peripheral immune system with rapid mobilization of immune effector cells (i.e. neutrophils, monocytes, T-cells) from the spleen [65, 67]. These mobilized effector cells can invade the neonatal brain through a disrupted blood-brain barrier and aggravate the existing injury [65, 67]. This concept has been confirmed and extended by our data in a preclinical model of neonatal HIE, showing that neuroinflammation mediated by microglia was associated with marked mobilization of the peripheral immune system and splenic involution [58]. These immunomodulatory changes were associated with an increased seizure burden and induction of white matter injury. In our studies, we administered MSC [58] and MAPC **(chapter 5)** at 1 h and/or 4 days after global HI since these cells have shown to possess both strong anti-inflammatory and regenerative capacities in CNS injury. These capacities are influenced by the host' microenvironment. With the first dose, we aimed to dampen the acute peripheral and cerebral inflammatory response which is considered as one of the first steps leading to EoP [1, 9]. Interestingly, we showed in our pre-clinical model that MSC induced persistent peripheral T-cell tolerance in vivo and reduced invasion of T-cells into the preterm brain following global HI, which might be a mechanism underlying the therapeutic effects of MSCs [58]. Ultimately, attenuation of the cerebral and peripheral inflammatory response, will prevent/inhibit subsequent cell death and injury in the brain.

Besides the immunomodulatory effects, we postulate that MAPC cell therapy promotes remyelination in a similar manner as MSCs, which have been shown to stimulate neural progenitor cells to differentiate towards the OL lineage and induce remyelination in vivo. Therefore, the second dose was administered 4 days after global HI to support repair of oligodendrocyte and neuronal injury, which is initiated in the sub-acute phase after HI-injury. We showed that MAPC cells prevented hypomyelination and

induced almost complete protection against PC loss at 7 days after HI. This is a very short period which makes protection a more likely mechanism than regeneration. In line, another group that studied the neuroprotective effects of stem cell based therapies in the preterm ovine fetus showed that these cells suppressed cerebral inflammation and protected white matter structures [68]. This study attributed these protective effects by indirect systemic and neuroimmunomodulatory effects. The neuroimmunomodulatory effects included the induction of a microglia phenotype switch towards a resting state, i.e. from a pro- inflammatory M1 phenotype to an anti-inflammatory M2 phenotype. Miron et al. showed that these M2 microglia enhance OL differentiation during remyelination in the brain [69]. Unfortunately, we cannot confirm the MAPC-induced switch towards a dominant, neuroprotective M2 phenotype since ovine-specific reagents to discriminate M1 from M2 microglia were not available at that moment. Recently, M2 specific markers (including CD 163) have been validated in fetal sheep studies which in future studies are useful to discriminate microglia phenotypes (unpublished). Altogether, we consider that especially the early administration of MAPC cell treatment is accountable for the neuroprotective effects observed in this study. Although one might question the clinical feasibility of the 1 hour gift, recent experimental and clinical data indicated that later (12-36 hour) administration of stem cells similarly protected the adult brain after stroke [70]. Based on these combined findings, we expect that administration of MAPC cells to neonates at a clinically more feasible time point (24 hours) after the HI event will result in comparable neuroprotective effects as shown in our current study. Clinically, the pathophysiological process of encephalopathy in preterm infants evolves over time and therefore we cannot exclude that late administration of MAPCs would still exert beneficial effects at a later stage of brain development. Therefore, we consider the timing of analysis as an important limitation for the assessment of regenerative effects of MAPC cells in our study.

Despite that immunomodulatory and regenerative effects have been shown, the underlying mechanism of action of stem cell therapies remain largely unknown. It was initially thought that the therapeutic action of stem cells relied on direct replacement of dead and injured cells. However, since the number of cells that reach the site of injury is minimal, there is marginal engraftment and short cell survival (72h), this theory was largely disregarded [59, 71]. In line, we and others could not identify MAPC cells

within the cerebral parenchyma after 7 days [59, 71]. Meanwhile, there was growing evidence that the pharmacological effects of stem cell therapy rely at least in part on paracrine mechanisms since studies showed comparable therapeutic effects of MSC-conditioned medium when compared to its cellular equivalent [72, 73]. Consistently, we have previously shown in our fetal sheep model of global HI that i.v. administration of **mesenchymal stem cell-derived extracellular vesicles (MSC-EVs)** resulted in partial protection against hypomyelination [74]. Remarkably, these therapeutic effects could not be explained by anti-inflammatory effects of the MSC-EVs such as seen after MSC treatment. This prompted us to focus on an alternative explanation for pharmacological effects of MSC-EVs, being restoring the injured BBB following global HI. There is accumulating evidence that the BBB around the second trimester becomes functional [75]. However, a global HI insult results in release of reactive oxygen species and excitotoxic molecules (direct/metabolic effects of HI) and cytokines released from the peripheral and local innate immune system (inflammatory component) leading to BBB dysfunction [76]. An increased permeability of the BBB results in infiltration of peripheral immune cells (e.g. macrophages, leukocytes, T-cells) that can aggravate white matter injury by the release of pro-inflammatory mediators. Therefore, strengthening or restoring maintenance of BBB integrity by enforcing endothelial cells could restrict the extent of white matter injury. In **chapter 6**, we observed that global HI negatively affects the integrity of the fetal blood-brain barrier (BBB). We provide substantial evidence that this reduced integrity is associated with an acute drop in **Annexin A1 (ANXA1)** expression in the cerebral vasculature. Annexin A1 is known as an endogenous regulator of BBB integrity in neurodegenerative diseases [77]. Moreover, using an in vitro model of fetal cerebral endothelial cells we demonstrated that targeting the ANXA1/FPR axis is effective in restoring the BBB integrity loss following oxygen and glucose deprivation. Importantly, Annexins, including ANXA1, are frequently found in the proteome of stem cells and their EVs [78]. Altogether, we conclude from our previous and current findings that the protective effects of MSC-EVs arise, at least in part, from their protective actions on the BBB which may be mediated by Annexin A1. This concept is supported by a recent study that demonstrated that the BBB integrity in a murine brain endothelial cell line was rescued by administration of human recombinant ANXA1 following β-Amyloid 1-42 (Aβ42)-induced BBB disruption [79]. Although our combined findings indicate that ANXA1-driven inhibition of cerebral inflammation is not a plausible explanation

for the pharmacological effects of MSC-EVs, we cannot rule out the possibility that microglia are part of the underlying working mechanism. More precisely, Solito et al. found that ANXA1 plays an important role in controlling non-inflammatory phagocytosis of apoptotic cells and promoting resolution of inflammation in models of Alzheimer's disease [80]. An Annexin-driven switch into M2 microglia that typically express such characteristics is a realistic scenario that warrants further investigation in a follow up study where neuroprotective effects of ANXA1 will be directly tested in vivo. Taken together, we and others show that the beneficial effects of stromal derived cells depend on a combination of actions of the intact cell and its secretome.

Future studies – personalized medicine – designing targeted stem cell therapies

The work presented in this thesis helps to unravel the time-dependent changes in the fetal brain following perinatal inflammatory stress. Apoptotic cell death, measured by the number of cleaved caspase-3 positive cells, is an important prognostic factor for neurological outcomes [81]. Importantly, whereas HI-induced cell death has been observed within 3 days after the HI insult [82, 83], we found increased apoptotic cell death following antenatal exposure to infection from 8 days post intra-amniotic LPS exposure **(chapter 3)**. These time dependent differences in the acute phase following a sterile or infectious inflammatory trigger are important determinants in defining a treatment regimen.

The data presented in this thesis indicate that defined windows of opportunities emerge following perinatal inflammatory triggers which are different following an HI insult compared to an infectious trigger. These important findings indicate a potential mismatch between the optimal timing and current clinical initiation of treatment regimens which might be an explanation for the reported inconsistencies of clinical trials. This may in particular be true for trials with EPO [84]. More precisely: the work

described in **chapter 3 & 6** shows a time-dependent and transient decrease of the pEPOR expression and endogenous ANXA1 content in the brain. Therefore, availability of a technology to detect the onset of inflammation *in utero* in a safe and non-invasive manner is key for the application of this concept. Moreover, every child that is born has its own "fingerprint" of risk factors potentially contributing to the development of EoP. This demands detection tools for the recognition of these distinct risk factors and a more individualized treatment approach. Correct timing of treatment initiation in relation to the nature and stage of injury is of great clinical importance in the near future.

Our studies and other preclinical studies primarily focused on single hit models with no standardized route, dose or intervention time of (cell-based or cell-derived) therapies. Timing and route of delivery for many treatments and dosage related effects are likely to play a role in efficacy and therapeutic potential. In experimental settings, several research groups have used intracranial delivery of neurotherapeutics [85, 86], however clinically a less invasive administration route is preferred [87]. Intranasal delivery is emerging as an effective administration method for especially cell based therapies which directly targets the brain, preventing loss of cells in the peripheral organs. Comparing intravenous delivery, which has the proposed advantage of reducing systemic inflammation as a key factor in initiating EoP, to intranasal delivery should be elucidated in future studies [56].

The best studied treatment currently in clinical trials is EPO. Although EPO is an endogenous factor expressed in the fetal brain, preclinical studies have shown that EPO treatment requires pharmacological, non-physiological doses to cross the blood-brain barrier to acquire a neuroprotective effect [88, 89]. Clinical trials still show great variability in used dosages of EPO which could explain the inconsistent results [84].

Until today, hypothermia is the only clinically available effective neuroprotective treatment for neonates at risk for brain injury. We know that hypothermia alters the time course of pathophysiologic events resulting in brain injury including energy failure and inflammation. Moreover, it modifies drug metabolism drastically. Therefore, designing new neurotherapeutic strategies must consider the pharmacological alterations induced by hypothermia and future studies should focus on these potential interactions of treatments.

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In conclusion, the nature of brain injury in preterm neonates is very complex, multi factorial and sensitive approaches for patient identification are essential. In addition, preclinical studies primarily focused on single hit models with no standardized route, dose or intervention time of (cell-based or cell-derived) therapies. This mismatch impedes development and or translation of neurological therapeutics for preterm infants, explaining the existing unmet clinical need. As such, future (pre) clinical studies should (1) address the multi-factorial nature of EoP, thereby allowing (2) proper patient stratification; (3) focus on dose, route and timing; and (4) explore synergistic approaches in which cell-based therapies are combined with therapies, such as EPO, melatonin, glucocorticoids, and hypothermia. Achieving the correct poise of interventions at the correct time in relation to the underlying pathophysiology and stage of injury will be a significant challenge in the next years.

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8

Nederlandse samenvatting

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Vroeggeboorte en inflammatoire stress rondom de geboorte zijn sterk geassocieerd met neurologische handicaps gedurende het gehele leven. In de afgelopen jaren is er uitgebreid onderzoek verricht om de onderliggende oorzaak en mechanismen die leiden tot deze hersenschade te identificeren om zo uiteindelijk nieuwe behandelingen te ontwikkelen. Echter, er blijven bepaalde essentiële vragen onbeantwoord waardoor deze resultaten maar in beperkte mate worden geïmplementeerd in de klinische praktijk. Het gevolg hiervan is dat tot op de dag van vandaag nog steeds veel vroeggeboren kinderen hersenschade ontwikkelen. In mijn thesis onderzoek ik het ontstaan van hersenschade na verschillende (gecombineerde) triggers, focus ik mij op de tijdsafhankelijke veranderingen in de hersenen van de vroeggeborene na deze triggers om zo het juiste tijdstip van behandelen beter te kunnen vaststellen en test ik nieuwe behandelingen.

EoP (hersenchade bij vroeggeborenen) heeft verschillende onderliggende oorzaken en ontstaansmechanismen. De twee meest belangrijke bijdragende factoren die ik onafhankelijk van elkaar heb bestudeerd in dit proefschrift zijn een intrauteriene ontsteking (chorioamnionitis) **(hoofdstuk 2-4)** en zuurstoftekort (globale hypoxie-ischemie) **(hoofdstuk 5 & 6)**. Om dit te bestuderen heb ik gebruik gemaakt van een preklinische groot proefdiermodel, het schaapmodel, welke de humane hersenontwikkeling tijdens de zwangerschap zeer nauwkeurig nabootst. In **hoofdstuk 2-4** heb ik gebruik gemaakt van intra-uteriene infectie/inflammatie modellen welke de klinische acute/chronische chorioamnionitis met foetale betrokkenheid (FIRS) nabootst. In deze hoofdstukken worden de tijdsafhankelijke veranderingen en processen in het premature brein in kaart gebracht na blootstelling aan een intra-uteriene infectie (chorioamnionitis). Chorioamnionitis is een polymicrobieel ziektebeeld en in ons model maken wij gebruik van verschillende inflammatoire stimuli inclusief Ureaplasma Parvum (UP), C. albicans en E. coli-derived Lipopolysaccharide (LPS) welke de meest voorkomende bacteriën en schimmels in chorioamnionitis vertegenwoordigen.

De eerste belangrijke bevinding van mijn werk is dat in de acute fase na blootstelling aan verschillende microbiële triggers (UP, Candida als LPS) een systemische immuunreactie optreedt die ik gekarakteriseerd heb door bepaling van het cytokine IL-6 in het foetale serum. Verhoogde systemische IL-6 levels is een essentiële marker voor het vaststellen van een foetaal inflammatory response syndrome (FIRS) welke in meerdere klinische studies is geassocieerd met neonatale mortaliteit en morbiditeit waaronder hersenschade inclusief cerebrale parese (CP). Onze data laat zien dat de IL-6 levels acuut en kortdurend verhoogd zijn na blootstelling aan intra-uteriene blootstelling aan inflammatie (UP, LPS) **(hoofdstuk 2 & 3).** Om de bepaling van IL-6 levels als klinische biomarker voor het ontstaan van hersenschade te gaan gebruiken is het belangrijk om het tijdstip van deze bepaling mee te nemen in het beslismodel. Daarnaast zullen studies zich moeten gaan focussen op het combineren van bestaande systemische inflammatoire markers (CRP, procalcitonine) of nieuwe biomarkers die het ontstaan van een intra-uteriene infectie kunnen bepalen om zo de neurologische uitkomst beter te kunnen voorspellen in de toekomst.

In **hoofdstuk 2** onderzoek ik de gevolgen van chronische intra-uteriene blootstelling aan Ureaplasma Parvum (UP) op het foetale brein. Ureaplasma species worden in vrouwen die te vroeg bevallen het meeste geïsoleerd uit het vruchtwater en placenta. Ondanks dat UP de meest voorkomende geïsoleerde microbe is bestaat er veel onenigheid over de rol van UP binnen de neonatale morbiditeit en mortaliteit. In mijn onderzoek laat ik zien dat chronische intra-uteriene blootstelling aan UP veranderingen in het foetale brein veroorzaken die op lange termijn zijn geassocieerd met een veranderde neurocognitieve ontwikkeling, waaronder verlies van astrocyten, epigenetische veranderingen en veranderingen in ontwikkelende witte stofcellen. Deze veranderingen kunnen verklarend zijn voor de geobserveerde veranderde neurologische uitkomsten op 2-jarige leeftijd in kinderen die geboren zijn en blootgesteld aan UP geïnduceerde chorioamnionitis.

Een volgende belangrijke bevinding laat zien dat in ieder infectieus/ inflammatoir model de systemische ontstekingsresponse (verhoogd IL-6) gevolgd wordt door een cerebrale response gekenmerkt door activatie van microglia, veranderingen in de witte stof maturatie en verhoogd aantal apoptotische cellen. Opmerkelijk is dat deze verhoogde celdood

na blootstelling aan een inflammatoire trigger relatief later ontstaat in vergelijking met na een hypoxisch-ischemisch insult **(hoofdstuk 3)**. Na blootstelling aan LPS zien wij een verhoogd aantal caspase-3 positieve cellen (marker voor apoptose) vanaf dag 8, waarbij dit na een globaal HI insult al vanaf dag 3 wordt waargenomen. Daarnaast wijzen de geobserveerde witte stof veranderingen op een maturatie arrest, een beeld dat naast het verlies aan witte stof als meest belangrijk onderliggend probleem gezien wordt in hersenschade van de vroeggeboren baby (EoP). Daarnaast maken deze veranderingen in de witte stof de baby vatbaar voor het ontwikkelen van neurologische handicaps gedurende het leven welke de DOHaD (developmental origin of health and disease) oftewel Barker hypothese ondersteunt. In mijn thesis worden verschillende oorzaken van deze veranderde witte stof maturatie als verklaring gevonden en onderbouwd waaronder afwijkende en overmatige microglia activatie, veranderingen in astrocyten populatie en epigenetische veranderingen. Echter een beperkende factor aan deze studies is de korte termijn follow-up. Lange termijn follow-up studies worden op dit moment uitgevoerd waarin de lange termijn gevolgen en de pathofysiologische processen onderliggend aan deze veranderingen bestudeerd zullen worden.

In **hoofdstuk 4** heb ik de effecten van Candida albicans op het brein bestudeerd aangezien chorioamnionitis niet alleen wordt beperkt tot bacteriële triggers. Hoewel C. albicans geïnduceerde chorioamnionitis maar zelden voorkomt, zijn de effecten hiervan op de foetus enorm schadelijk. Ook in dit model zien wij een vergelijkbaar patroon aan veranderingen (verhoogd IL-6, cerebrale ontsteking en witte stof veranderingen). Een belangrijke bevinding vanuit deze studie is dat een eenmalige gift van intra-uteriene Fluconazole (antimycoticum) wel in staat is om neonatale sterfte te voorkomen maar niet de cerebrale ontstekingsresponse en potentieel schade op de lange termijn tegen gaat. Deze bevindingen pleiten ervoor dat een behandeling naast antimicrobiële/bacteriële eigenschappen ook immunomodulatoire/anti-inflammatoire effecten moet hebben.

Behandelingen

Therapeutische hypothermie is tot op heden de enige behandeling die hersenschade (deels) kan voorkomen die clinici voorhanden hebben voor pasgeboren baby's. Echter, 40% van de behandelde kinderen heeft ondanks hypothermie nog steeds afwijkende neurologische uitkomsten op de latere leeftijd. Daarnaast heeft hypothermie in vroeggeboren kinderen meer

bijwerkingen en wordt afgeraden in deze kwetsbare groep kinderen. Meer recent onderzoek laat ook zien dat in de aanwezigheid van een infectie (chorioamnionitis) hypothermie helemaal niet effectief is en mogelijk zelfs nadelige gevolgen heeft. De noodzaak voor een nieuwe behandeling om EoP te voorkomen/behandelen is dus groot. Stamcellen zijn mogelijk een goede kandidaat hiervoor. Stamcellen bezitten zowel regeneratieve als immunomodulatoire eigenschappen. In eerder onderzoek heeft onze groep laten zien dat beenmerg afkomstige stamcellen (MSCs, MAPCs) in staat zijn om cerebrale ontsteking, witte stofschade en functieverlies als gevolg van zuurstoftekort kunnen voorkomen in het schapenmodel.
In **hoofdstuk 5** laat ik zien dat deze MAPCs ook in staat zijn om het cerebellum (kleine hersenen) te beschermen tegen zuurstofgebrek rondom de geboorte. Schade aan het cerebellum speelt een toenemende belangrijke rol in de ontwikkeling van (niet-motorische) problemen na vroeggeboorte. Na zuurstoftekort rondom de geboorte (hypoxie-ischemie) ontstaat er een ontstekingsreactie, verlies van witte stof en verlies van belangrijke neuronen (purkinje cellen) in het cerebellum. Deze veranderingen passen bij een vertraagde of achterblijvende ontwikkeling van het cerebellum en worden geassocieerd met EoP. Ik laat zien dat MAPCs deze veranderingen kunnen voorkomen en hiermee dus problemen op latere leeftijd worden voorkomen.

In **hoofdstuk 6** onderzoek ik het onderliggende mechanisme van deze stamceltherapie. De mechanismen waarop stamcelen het immuunsysteem kunnen beïnvloeden zijn namelijk grotendeels onbekend. Een voorgesteld mechanisme is door uitscheiding van extra-cellulaire blaasjes (EVs) waarin immuun modulerende moleculen verpakt zitten. In ons schaapmodel hebben wij eerder laten zien dat EVs afkomstig van stamcellen (MSC-EVs) in staat zijn om de duur en aantal convulsies te verminderen na zuurstoftekort. Daarnaast was er een neiging tot witte stof bescherming. Echter, in tegenstelling tot de stamcel zelf kon dit beschermend effect van de EVs niet verklaard worden door anti-inflammatoire effecten. Als alternatief werkingsmechanisme laat ik zien in deze studie dat MSC-EVs in staat zijn de bloed-breinbarrière (BBB) te herstellen na een periode van zuurstoftekort rondom de geboorte. Het is algemeen bekend dat de BBB na zuurstoftekort beschadigd raakt waardoor schadelijke immuuncellen uit het bloed de hersenen kunnen binnendringen en hier bijdragen aan de ontstekingsresponse en witte stofschade. Daarnaast laten wij zien dat deze

verstoring in barrière gepaard gaat met een vermindering van AnnexineA1 (ANXA1) expressie in het brein. ANXA1 staat bekend als een regulator van de bloed-hersenbarrière en speelt ook een rol in het ontstaan van een beschadigde barrière in ziekten als Parkinson en Multipele Sclerose. In een kweekmodel toon ik aan dat door het toedienen van zowel MSC-EVs als ANXA1 de bloed-hersenbarrière wordt hersteld na een periode van zuurstoftekort. Deze bescherming van de BBB komt door signalering van ANXA1, via de ANXA1/FPR-axis welke het tight junction complex van de endotheelcellen versterkt. Concluderend, het beschermende effect van MSC-EVs kan (deels) worden verklaard door het beschermende effect van de bloed-hersenbarrière, welke wordt gemedieerd door ANXA1.

Tot slot heb ik in **hoofdstuk 3** ingezoomd op de regulatie van het receptor systeem van een ander potentieel neuro-farmacologische toepassing namelijk het al bekende cytokine Erytropoëtine (EPO). Zowel uit deze studie, als ook uit de data van het ANXA1/FPR receptor systeem **(hoofdstuk 6)**, blijkt dat er op specifieke tijdstippen na een pro-inflammatoir insult in utero, er veranderingen ontstaan in deze complexen welke inzicht verschaffen over mogelijke timing van start van behandelingen. Zo ontstaat er na blootstelling aan een pro-inflammatoir insult over de tijd een depletie ontstaat van het endogene ANXA1. Tevens hebben we een verminderde activiteit van de EPO-receptor aangetoond. Deze resultaten vormen een mogelijke verklaring voor de wisselende uitkomsten van klinische studies waarin EPO als mogelijk behandeling in pasgeborenen werd getest. Tevens hebben we voor het potentieel klinisch gebruik van AnxA1 een “optimal window of opportunity” blootgelegd.

De toekomst

Hersenschade bij vroeggeborenen is multifactorieel en complex en kent een zeer heterogene patiëntenpopulatie. Ieder kind heeft een eigen “vingerafdruk” van risicofactoren die een rol spelen in het ontstaan en verloop van mogelijke hersenschade. Dit behoeft een individuele aanpak wat betreft de behandeling (personalised medicine). In mijn onderzoek breng ik tijdsafhankelijke veranderingen in het premature brein in kaart na inflammatoire blootstelling in utero. Belangrijk hierin is dat ik laat zien dat er mogelijk een mismatch bestaat tussen het optimale moment waarop behandeling het meest effect zou zijn en het tijdstip waarop er in de kliniek daadwerkelijk behandeling wordt gegeven. Om een individuele aanpak

te implementeren in de kliniek is stratificatie middels betrouwbare biomarkers een vereiste. De timing en het type insult spelen hierbij een sleutelrol. Afhankelijk van de voorgeschiedenis van de pasgborene kan een op maat beleid worden afgesteld. Interventies middels stamcellen, Annexine A1 en EPO (of een combinatie hiervan) zijn in dit kader veelbelovend.